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IN VITRO EXPERIMENTS WITH GRANULATION TISSUE ON THE MATURATION OF COLLAGEN

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Two stages can be defined in the formation of insoluble collagen fibres from tropocollagen macromolecules: (1) the alignment of the tropocollagens, which usually is accompanied by precipitation, and (2) the maturation of the precipitated collagen into insoluble fibres. Much attention has been paid to the mechanism of the first phase (*Schmitt* 1959), but the second is almost unknown, even if much work has been devoted to the intermolecular cross-links of collagen (for general reference, for example, *de la Burde, Peckham & Veis* 1963). It is quite established, that during the development of the collagenous tissue, the soluble collagen is gradually converted to less soluble forms and that during the involution the reverse order applies.

The purpose of this work was to explore the possibilities to study the insolubilization of collagen *in vitro*. The method was to incubate *in vitro* various preparations of granulation tissue and analyze the solubility fractions on hydroxyproline. Two kinds of experiments were made: (1) without the addition of collagen and the results refer to endogenous collagen and (2) with additions of soluble collagen from the same species either in clear solution or in suspension of precipitated collagen where the tropocollagen macromolecules were in proper "register" already.

EXPERIMENTAL

Preparation of the soluble collagens. The procedure was modified from the paper by *Gross* (1958a). The skins of 10 guinea pigs (weighing 193–365 g) were pooled (together 220 g). The hair and subcutaneous tissue were removed and the material was first minced with meat grinder and finally homogenized with Waring Blendor into 500 ml of 0.45 *M* NaCl. The resulting suspension was shaken at +2° C for 16 hrs.

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The supernatant was obtained by centrifugation (for 60 min. at 50,000×g) and filtration through No. 2 sintered "Jena" glass filter and finally dialyzed overnight against pH 7.6 phosphate buffer (ionic strength = 0.4). Trichloroacetic acid was added (as 25 per cent solution, w/v, in 0.45 *M* NaCl) in small portions until the solution contained about 2.5 per cent trichloroacetic acid and the pH was 3.5. The mixture was left to stand for 30 min. and then centrifuged. The supernatant was dialyzed against the same phosphate buffer, and the fluid remained clear. The soluble collagen was precipitated with absolute ethanol, which was added slowly to the concentration of 14 per cent (v/v). The mixture was centrifuged after 24 hrs. The precipitate was dissolved into 40 ml of 5 per cent acetic acid. All the manipulations were performed at +4° C. The final solution contained hydroxyproline about 5 mg/ml or about 3.5 per cent of collagen.

For the experiments 1.4 ml of this solution in acetic acid was diluted with 3.6 ml of the Krebs 4A-solution (*Green & Lowther* 1959), and dialyzed for 72 hrs. at +2° C against 100 ml of the same Krebs 4A-salt solution containing 22.4 mM glucose. The outside fluid was changed daily. The precipitate, which contained about the half of the collagen, was removed by centrifugation (4000 r.p.m., 30 min., in cold room) and the clear supernatant was used in the experiments.

To check whether the alignment of the tropocollagen macromolecules was significant for the action of the granuloma homogenate, acid-soluble collagen of guinea pig skin (prepared by Dr. *J. Pikkarainen*) was precipitated by dialysis against 20-fold volume of Krebs 4A-glucose solution, which was changed daily, for 4 days in the cold room. The precipitate was suspended into 100-fold volume (v/wet weight) of the same Krebs 4A-glucose solution and this suspension was used instead of collagen solutions. With electron microscopy it was ascertained, that the collagen was present in clean fibers with the normal periodicity.

Experiments with carrageenin granuloma homogenates. Commercial samples of carrageenin were used as 1–1.5 per cent (w/v) aqueous solution for the production of the granulation tissue in the guinea pig (average weight 410 g). Five ml of the carrageenin solution was injected subcutaneously in the abdominal flanks and the granulomata were harvested after several time intervals. If not stated otherwise, seven day old granulomata were used.

The granulomata (15–40 g) were dissected and homogenized with an equal amount (v/wet weight) of the aforementioned Krebs 4A-glucose solution, first with a Bühler-homogenizer (3 min. with full speed, nominally 50,000 r.p.m., cooled with water at +7° C) and finally with Potter-Elvehjem homogenizer (A. H. Thomas, Cat. Size C), by passing the teflon pestle six times to the bottom. Nothing was removed from the homogenate, which, as a rule, was used immediately. For each batch of homogenate the granulation tissue was pooled from two guinea pigs and the respective control experiments were always carried out with the same homogenate.

For the incubation 2 ml portions of the homogenate were taken. The exogenous soluble collagen was added either as a clear solution or as a suspension of fibrous precipitate, both in Krebs 4A-glucose solution. The amounts of added collagen were 5 mg (in 1 ml of solution) or 10 mg (in 1 ml of suspension), which had been found suitable in the preliminary experiments.

The suspensions of homogenate, with or without added collagen, were incubated at + 37° C for various time intervals in Gallenkamp Shaking Incubator (No 6990) in erlenmeyer-flasks and the controls were kept in cold room (+6° C). After incubation the flasks were chilled in ice-water and stored in the cold room for several hours. The contents of the flasks were homogenized with Potter-Elvehjem homogenizer (A. H. Thomas, size A, nominal speed 2000 r.p.m.) by passing the pestle six times to the bottom. At this stage 6–8 ml of Krebs 4A-glucose solution was added, also to the non-incubated controls, which were not homogenized at this stage.

Experiments with other preparations of granulation tissue. In preliminary experiments the granulomata were induced with viscose cellulose sponge *(Viljanto & Kulonen* 1962). They are not presented in detail, because the carrageenin granulomata were preferred. From the latter the preparation of the homogenates was easier and the granulomata contained larger fraction of soluble collagen, even if the production of the granulomata was more variable.

In the earlier phase some experiments were performed also with granuloma slices, which were cut with a thin knife at 0.3 mm thickness. Also this form of preparation was abandoned, because the homogenate could be measured in more reproducible amounts.

Fractionation of the homogenates. The supernatant was obtained by centrifugation for 30 min. at 3000 r.p.m. in the cold room. The sediment was rehomogenized with Potter-Elvehjem homogenizer as above into 6–10 ml of the desired solvent (salt or acetic acid solution). The homogenates were kept in the cold room overnight and then centrifuged as above. A control experiment was carried out to check the effect of a more powerful centrifugation. At 50,000×g about 22–26 per cent ($n = 6$) of the collagen, left in the 0.45 *M* NaCl-soluble fraction at the regular centrifugation, was sedimented. However, the proportion was remarkably constant and therefore the significance of the results does not seem affected. The sediment from the acetic acid-soluble fraction was negligible (about 4 per cent). In the series in which precipitated collagen had been added, the centrifugation was carried out with a refrigerated MSE centrifuge (9000×g).

Analyses and calculations. The samples were hydrolyzed in sealed tubes in 6 *N* hydrochloric acid overnight at +105° C. The hydroxyproline was analyzed by the method of *Neuman & Logan* (1950), always in duplicate. The standard curve was checked when new reagents had been prepared at intervals of about two weeks.

The amount of the total collagen varied even in parallel samples, because of the difficulty in measuring the homogenate, and the following way of presentation was adopted. From the usual three parallel incubations absolute average amounts for each fraction were calculated. From the sum of these averages a correction coefficient was calculated to make the data of incubated samples comparable with the respective non-incubated samples.

RESULTS

Changes in the endogenous collagen of the granulation tissue. The proportional amounts of the various collagen fractions during the development of the granulomata are collected in Table 1. The peak of the collagen content is reached on the 10th day. The proportions of the fractions are rather constant, except on the 4th day, when the amount of collagen is still very small. The amount of insoluble collagen never exceeds three fourths. The findings are in reasonable agreement with the study by *D. S. Jackson* (1957), who observed the peak of collagen content on the 14th day. According to his studies the proportion of the acid-soluble fraction increased towards the involution of the granuloma.

The changes in the soluble hydroxyproline-containing fractions at incubation of the homogenates are shown in Table 2. The homogenate of 15-day-old granulation tissue differed from the general pattern. In spite of the large variation between the individual experiments, it seems justified to state that the soluble collagen decreased at the incubation, mainly in the fractions which are soluble into 0.45 *M* NaCl and into 3 per cent acetic acid. The fraction, which was soluble in Krebs 4A-solution did not change so much, except in the case of 15-day-old granuloma, where it increased at the expense of the other soluble fractions and the total soluble fraction did not decrease at all during the incubation. The prolonged incubation for 4 hrs. did not obviously change the result of one hr. incubation.

Experiments were carried out also with homogenates stored for 7 and 13 days. In both experiments especially the 0.45 *M* NaCl-soluble and acetic acid-soluble fractions decreased, but the results were irregular, because the amount of the insoluble collagen decreased in the case of 7 days old granuloma.

TABLE 1

The Hydroxyproline-Containing Fractions of Carrageenin Granulomata during the Development.

Age of the granulation tissue	Total hydroxy-proline μg	Fraction of hydroxyproline, %			
		Soluble in Krebs 4 A solution	Soluble in 0.45 *M* NaCl	Soluble in 3% acetic acid	Insoluble
4 days	155	37	14	9	40
6 days A	612	15	8	10	67
6 days B	1210	11	12	11	66
7 days	1410	10	8	8	74
10 days	1640	10	8	8	74
15 days	1200	11	12	9	68

The figures are derived from the averages of duplicate or triplicate determinations.

TABLE 2

Changes of the Soluble Hydroxyproline-Containing Fractions of Granulation Tissue at Incubation.

Age of the granulation tissue	Type of preparation	Total soluble hydroxy-proline initially μg	Change in μg			
			Total	In the fractions, soluble in		
				Krebs 4 A solution	0.45 *M* NaCl solution	3% acetic acid
4 days	homog.	93	—10	—3	—4	—3
6 days A	homog.	200	—37	—4	—20	—13
6 days B	homog.	420	—35	—2	—16	—17
7 days A	homog.	360	—9	+7	—21	+5
7 days B	homog.	80	—20	—9	—7	—4
10 days	homog.	430	—50	+2	—17	—35
15 days	homog.	380	+3	+33	—8	—22
7 days C	slices	190	—26	—19	—3	—4
6 days C	homog.	120	—30	0	—2	—28
6 days C*	homog.	120	—28	0	—4	—24

* incubated for 4 hrs.

Duration of incubation 1 hr. Figures are derived from averages of triplicate experiments. No exogenous collagen was added.

Two series of experiments were carried out in the presence of β-aminopropionitrile (0.01*M*). The first two experiments were made with homogenates of 6 and 7 days old granulomata. In short, they indicated that the presence of β-aminopropionitrile hydrosulfate prevented the effect of incubation. However, when the experiment was repeated with 0.001 *M* β-aminopropionitrile and the hydrosulfate was carefully neutralized, no effect of the presence of β-aminopropionitrile was observed.

Changes in added collagen. From Table 3 it is evident that the presence of the homogenate during the incubation induced an increase in the NaCl- and acetic acid-soluble fractions of added collagen, even if the collagen had been added as a precipitated suspension.

TABLE 3

Effect of Incubation of the Granulation Tissue on Added Precipitated Collagen.

Solubility	Incubated						Not incubated
	With homogenate			Without homogenate			
	Exp. I	Exp. II	Average	Exp. I	Exp. II	Average	
Krebs 4A-solution	58.7	74.3	*66.5*	56.9	65.3	*61.2*	*18.4*
0.45 *M* NaCl	8.9	5.7	*7.3*	0.6	0.9	*0.7*	*1.6*
3 % acetic acid	19.0	9.0	*14.0*	13.0	5.4	*9.2*	*19.5*
Insoluble	13.5	11.0	*12.2*	29.5	28.4	*28.9*	*60.4*

The experimental figures are in per cent of the total hydroxyproline. Two independent sets of experiments (designated I and II) were carried out. The figures are derived from the averages of three incubation experiments. Time of incubation 1 hr.

Especially the salt soluble fraction was increased. The incubation itself caused a large increase of the "Krebs 4A-soluble" supernatant fraction, presumably because of denaturation of the collagen. This portion was not largely influenced by the homogenate, but the insoluble fraction decreased markedly, if homogenate had been present. Most of the hydroxyproline in the Krebs 4A-soluble fraction is in the non-diffusible form. The proportion of the diffusible hydroxyproline was not affected by the presence of homogenate.

Several experiments were made with the addition of the soluble collagen as a solution in Krebs 4A-glucose. Here will be presented only those which were made in triplicate (Table 4). From the data it is evident, that also in this case, the presence of the homogenate enhanced the proportional amount the fraction, soluble in 0.45 *M* NaCl, and decreased that which is soluble in Krebs 4A-solution. In the less controlled experiments the increase in the salt-soluble fraction was also found, as a rule.

TABLE 4

Effect of Incubation of the Granulation Tissue on Added Collagen Solution.

Solubility	Incubated							
	With granulation tissue				Without granulation tissue			
	Exp. I	Exp. II	Exp. III	Average	Exp. I	Exp. II	Exp. III	Average
Krebs 4A-solution	34.7	13.1	15.4	*21.1*	40.4	30.1	28.9	*33.1*
0.45 *M* NaCl	58.3	80.0	41.3	*59.8*	40.9	60.8	34.0	*45.2*
3% acetic acid	5.9	6.8	26.3	*13.0*	16.7	4.8	32.7	*18.1*
Insoluble	1.0	...	16.9	*6.0*	2.1	5.3	4.6	*4.0*

The figures are in per cent of the total hydroxyproline. Three independent sets of experiments were made, I and II with sponge granuloma homogenate, III with carrageenin granuloma homogenate. The figures are averages of three incubation experiments. Time of incubation 1 hr.

DISCUSSION

Nature of the changes. These experiments have some bearing on the work of *Gross* (1958b). He studied the reversibility of the heat gelation of various preparations of soluble collagen and found that especially the crude extracts were partly insoluble after heating for various periods. He suggests "that the steadily diminishing solubility of precipitated fibrils with time is, at least in part, a result of the interacting collagen molecules finding their most stable state of association". It is also a common experience that the amount of soluble collagen decreases *in vitro* in many tissues with time and that the precipitates of soluble collagen are not always fully soluble. The present experiments could be interpreted in the same terms, but it should be noted that the decrease in the solubility decreases to a certain limit only and that there seems to be a kind of equilibrium between the collagen fractions, which is especially manifested in Table 1.

It remains to be investigated whether these changes are purely physicochemical, depending on the precipitation with non-collagenous materials or on improved alignment, or whether they involve enzymatic activity.

It is obvious that part of the added collagen is denatured by the incubation, but not degraded to diffusible form (Table 3). The presence of homogenate induces the disintegration of the insoluble form to salt-soluble forms. What happens in the terms of chemical bonds, is not known. *Frankland & Wynn* (1962) and *Barsky & Farrison* (1963) have shown that liver, especially its microsomal fraction, contains an enzyme-like principle, which breaks down collagen, as has been demonstrated also by *Nagai, Lapiere & Gross* (1963) with other tissues. Homogenate may be assumed to contain something which penetrates between the collagen macromolecules and thus renders the fibres soluble.

At incubation the added pure fibrous collagen and the endogenous collagen of the granuloma itself changed in different manner. On this basis it may be further assumed that in the natural fibrillogenesis some non-collagenous matter is significant (cf. *Partington & Wood* 1963). When the homogenates were prepared, the endogenous soluble collagen was not converted into solution at all, but retained the natural configuration *in situ,* which may be significant in the normal maturation.

Are changes developing in the intra- or intermolecular linkages at incubation? As regards endogenous collagen the question is thus far impossible to settle experimentally, because the soluble collagen is only a very minor fraction of the accompanying soluble proteins and methods have first to be developed for the analysis of the subcomponents of collagen in the extracts of granulation tissue.

The study of the changes in the added collagens is more readily practicable, but thus far the sedimentation analysis (by Dr. *T. Nikkari*) hat not provided other results than non-defined changes in the α- and

β-fractions during incubation with tissue homogenate. The interpretation of the experiments is difficult also because the added collagen partly precipitates during the incubation at +37° C.

SUMMARY

The changes in the solubility of collagen were studied by incubation experiments with granulation tissue preparations.

The proportions of the collagen fractions (soluble in salt solutions or in acetic acid) were fairly constant during the growth of the carrageenin granulomata for up to 15 days.

The soluble collagen fractions, which were present in the granuloma homogenate itself, decreased at the incubation. The collagen, which had been added either in clear solution or in suspension of precipitate, behaved differently at the incubation with granuloma preparations. The presence of the homogenate increased the salt-soluble fraction. In addition, the insoluble fraction of the precipitated collagen decreased markedly.

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